PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G01N 15/00, B04B 5/04, 7/02, G01N 33/48	A1	(11) International Publication Number: WO 99/23471 (43) International Publication Date: 14 May 1999 (14.05.99)

(21) International Application Number: PCT/EP98/06865

(22) International Filing Date: 29 October 1998 (29.10.98)

(30) Priority Data:
MI97A002457 31 October 1997 (31.10.97) IT

(71)(72) Applicant and Inventor: SITAR, Giammaria [IT/IT]; Corso Partigiani, 32, I-27012 Certosa di Pavia (IT).

(74) Agent: GERVASI, Gemma; Notarbartolo & Gervasi S.p.A., Corso di Porta Vittoria, 9, I-20122 Milan (IT).

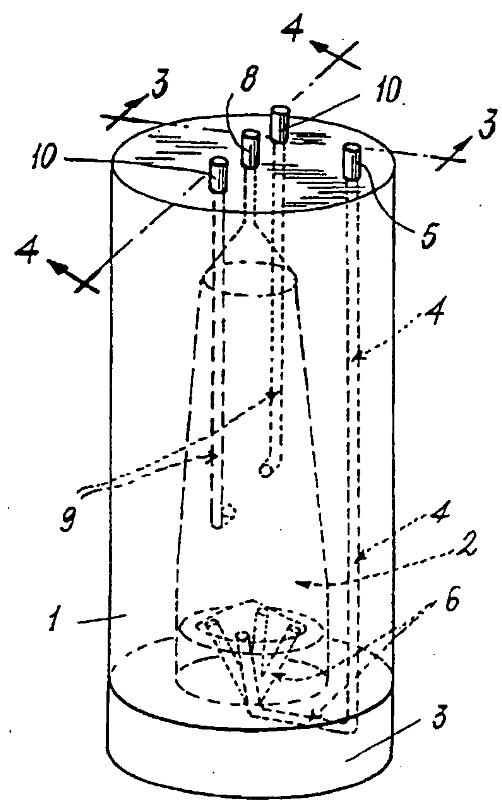
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DEVICE AND METHOD FOR THE SEPARATION OF HUMAN OR ANIMAL CELLS OF DIFFERENT DENSITIES FROM CELLULAR DISPERSIONS WHICH CONTAIN THEM

(57) Abstract

The invention concerns a device and a method which allow the efficacious and rapid separation of organic cellular populations of different densities, from the dispersions which contain them.



FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

DEVICE AND METHOD FOR THE SEPARATION OF HUMAN OR ANIMAL CELLS OF DIFFERENT DENSITIES FROM CELLULAR DISPERSIONS WHICH CONTAIN THEM

Summary

The invention concerns a device and a method which allow the efficacious and rapid separation of organic cellular populations of different densities, from the dispersions which contain them, said device comprising an elongated chamber whose cross section decreases from the base towards the top of the device and said method comprising the introduction of a dispersion of a cell population into said chamber of the device previously filled with a liquid whose density varies from a maximum, near the device base, to a minimum near the top, subjecting the device with the cellular dispersion to centrifugation and collecting the separated fractions by introducing a very dense water immiscible liquid at the base of the separator device while simultaneously pumping a gas at the same pressure as said very dense liquid, to the device top so that the layered cellular fractions inside the chamber are expelled through at least one hole situated at an intermediate level of said chamber length.

Prior art

The invention refers to both a device and method for the separation of human or animal cellular fractions of different densities, from cell populations present in cellular dispersions.

It is known that human or animal cells have differing densities (generally between 1.04 g/cm3 and 1.1 g/cm3) and that each cell type has its own characteristic density.

It is also known that it is often important and necessary to separate differing cell types for possible concentration or purification of the same, from a dispersion of a heterogeneous population of cells. This is fundamental in making the biochemical and functional analyses possible on both normal cells and neoplastic populations.

The finer and more careful the separation of different density cells, and the more rapid is the separation operation then the data obtained will be equally good and useful, with consequent benefits to the practical procedure.

If one considers, for example, that certain types of cells are present in the blood or human tissues in extremely reduced quantities, it is evident that from their effective and rapid identification, the possibility of chemical, biochemical or medical intervention, otherwise impossible or only hypothetical, could follow.

The most advanced cell separation technique in current use employs a separator device consisting of a casing with an internal cavity. The latter is laterally delimited by tapered surfaces, with the greatest cross section near the end or base from which a tube or duct end is connectable to a peristaltic pump. The cavity is tapered towards the opposite end or top of the casing, which has an outlet hole. Initially, a liquid is introduced into the base cavity of the separator device using the peristaltic pump and a gradient mixer. The liquid density slowly increases as the quantity of liquid in the cavity increases, until filling it. Thus, the separator device cavity is filled with a liquid which is practically made up of successive and continuous strata of liquid, increasing in density from the top to the base of the device cavity. At this point, the liquid supply is suspended and a dispersion of the cellular population to be separated into homogeneous cellular fractions by density is introduced through the hole in the top, until the upper cavity section is filled: at the same time as the dispersion introduction, a corresponding volume of the liquid first introduced is drawn off from the separator device base, so that the cellular population dispersion can fall into the device cavity.

The separator device is then closed in a centrifuge, with the rotation axis perpendicular to the separator device axis and near its top, during centrifugation. In the centrifuge, a very large force is transmitted to the liquid closed in the device (for example 400g), resulting in the distribution of the cellular population dispersion in the liquid in the form of cellular bands (or strata) of differing densities.

The separator device is then removed from the centrifuge and a very dense water immiscible liquid is introduced into the base causing the expulsion (from the hole in the top of the same device) of the fractionated cellular dispersion as described: the desired number (for example 10 or 12) of distinct dispersion fractions with an increasing density gradient are extracted through the separator device top, the cells present in each fraction having different densities to the adjacent fractions . The device and the methodology briefly outlined above, are described in detail by Giammaria Sitar and Piermaria Fornasari in HAEMATOLOGICA, Vol. 74, N1, Jan-Feb. 1989; and by Pretlow II TG and Pretlow TP eds. Cell Separation: method and selected applications, New York, Academic Press, 1982 (5 volumes).

The recovery system of the different separated cell fractions described above, has its limits, however, and presents some serious problems. Infact, the collecting of the different and subsequent cell fractions is carried out through a small hole in the separator device top, under the push of a dense liquid which is pumped in at the device base. This causes a hydrodynamic disturbance in the different adjoining strata where the cell density is different, and causes the widening (well known to field technicians) of the strata or bands where the different density cells are collected or distributed, resulting in the contraction of the liquid strata separating the bands where are collected the cells of mainly homogeneous density and different from band to band, which can even lead to the remixing of the various cellular fractions that were separated during centrifugation. This phenomenon is more serious and unacceptable, the narrower the bands, in which the cells have been collected, and the smaller the distance separating one cell from another.

In order to try and minimise these problems, the pressurised liquid at the base of the separator device is introduced with extreme slowness, a fact which leads, however, to long and unacceptable times necessary for the collection of the different cellular fractions. See the publications of Giammaria Sitar and Piermaria Fornasari already mentioned; and TULP, Anal. Biochem. 1981, vol. 117, pages 354-365.

Brief description of the drawings

- Figure 1 is a schematic view of a known separator device of cells;

- Figure 2, represents, a schematic view of a separator device according to the invention of which:

- Figures 3 and 4 are longitudinal sections of the device according to the lines 3-3 and respectively 4-4 of Figure 2.

Description of the invention

The main aim of the present invention is that of finding a device and method which allow the easy and very clear collection of cellular fractions which have been separated in the device after centrifugation.

Another aim is that of allowing the recovery of the different cellular fractions in shorter times than is currently possible, making the collection of useful data possible, which would otherwise have proved useless.

The above aims, along with others, are satisfied with a separator device with a base and top, containing an elongated chamber, whose cross section decreases from the base towards the device top, the latter containing at least a first channel, one end of which opens onto the inside of the said chamber near the said base, the other end connectable to a pressurised liquid source and a second channel, one end of which opens into the same chamber corresponding to the device top, characterised by the fact that in the said device there is at least an additional channel, one end of which opens along the said chamber length, the other end opening onto the exterior of the device.

The invention also concerns the separation method of cellular fractions from dispersions of human or animal cell populations, in which, first of all, the dispersion of a cell population is introduced into the separator device chamber, previously filled with a liquid whose density varies from a maximum near the device base and a minimum near the top. The device is then subjected to centrifugation so as to distribute the cells into distinct fractions with differing densities, the method being characterised by the fact that the separated fraction recovery occurs by introducing, in the device base, a very dense, water immiscible liquid while simultaneously pumping a gas to the top (at the same delivery

pressure as the very dense liquid), so that the layered cellular fractions inside the chamber are expelled through at least one hole situated in an intermediary zone along the said chamber length.

It was found, that proceeding in the above manner the cell bands, which were found layered in the separator device, were compressed after centrifugation (instead of expanding, as happens with the known method) in the same chamber, in a way that the various bands could be recovered separately in a clear manner, being expelled from the chamber (through the holes located laterally to the same chamber) without undergoing any hydrodynamic disturbance, i.e. there is no cell band remixing with those immediately adjacent.

It was also ascertained that, by proceeding in the manner described, the various cellular fractions could be separated and recovered at greater speeds than with the known technique, and cellular fractions are not mixed, as already mentioned.

In order to make clearer both the device structure and the invention procedure, there will now follow the description of a potential application, as an unlimiting example, with reference to the enclosed figures.

Reference is made, first of all, to Figure 1 which illustrates a separator device of known structure made of an elongated casing 1 with an internal bell cavity 2 delimited by a substantially conical surface (dotted lines) with a greater cross section towards its base (with respect to the diagram) where there is the base 3 body affixed, which seal closes the cavity 2. Casing 1 is crossed by a duct 4 from whose upper end, a tube 5 extends, whose lower end opens inside cavity 2 through the channels 6 located in base 3 casing which is fixed to casing 1 in a suitable way, for example, with a series of bolts (not shown) or screw connection.

According to the present invention near the base of the device a flow deflector 11 is provided to disperse evenly the incoming fluid arriving from the channels 6, through the entire cross section of the inside chamber 2.

Cavity 2 is tapered towards the top of casing 1 which is crossed by a duct 7 linking the cavity with tube 8.

When one wishes to use the illustrated device, cavity 2 is filled, in the known way using tube 5, duct 4 and channels 6, with a liquid whose density gradually increases as it is introduced into the cavity, until filling it.

Thus cavity 2 is filled with a liquid which is practically made up of successive and continuous strata of liquid with increasing densities from the top to the base of the device. The liquid supply, through tube 5, is then stopped and the cellular population dispersion to be separated into homogeneous cellular fractions by density is introduced in cavity 2, while a corresponding volume of liquid is drawn off through tube 5.

The separator device is then placed in a centrifuge at high speed until the distribution of the cellular population dispersion in the liquid, in form of cellular strata of different densities, is obtained.

At this point, a very dense water immiscible liquid is introduced into cavity 2, through tube 5, causing the expulsion of the cellular dispersion fractionated in the manner described from tube 8 collecting the desired number of distinct cellular dispersion fractions of increasing density gradient.

The recovery system of the different cell fractions described above with reference to Figure 1, presents the serious problem that the expulsion of the differing cellular fractions by tube 8 occurs entirely with the push of the dense liquid coming out from channels 6 at the device base, with the consequent well known contraction or thinning of the liquid strata separating the bands where are collected the cells of homogeneous density but different from band to band, (resulting in difficulties of separate collection of the different bands). This can lead to serious and unacceptable remixing of the differing cellular fractions that were separated during centrifugation.

To minimise the above problem, the introduction of the liquid at the base of cavity 2 is carried out with extreme slowness, which means long times are necessary for the recovery of the different cellular fractions which is extremely serious as known to the field technicians.

Figures 2 to 4 illustrate a separator device according to the present invention.

This separator device is similar to that in Figure 1: for clarity, the same reference numbers used in Figure 1 have been adopted for Figures 2 to 4 to illustrate the various parts.

The separator device of Figures 2 to 4 is different from that of Figure 1 for the fact that two supplementary ducts 9, are located in casing 1 (in reality, there could be more than two, or even one) from which a tube 10 extends from the upper end, whose lower end opens into the inside of cavity 2 halfway along its length.

The filling and centrifugation procedures of the device in Figure 2 to 4 have already been described with reference to Figure 1 and will not therefore be repeated here. The recovery stage of the different separated cellular fractions from the device is very different.

In fact, the collection of the separated fractions occurs by introducing a very dense water immiscible liquid at the base of cavity 2 (as in the known technique) while simultaneously pumping a gas (for example, air) through tube 8 to the top at the same pressure as the dense liquid, so that the layered cellular fractions inside cavity 2 are compressed and forced out through ducts 9.

It was found, proceeding in the above manner, that not only the recovery of the different cell bands which were layered in cavity 2, is performed at a much higher velocity than was possible with the known technique, but also happens so that the bands are compressed in cavity 2 (instead of expanding, as in the known technique) and can therefore be collected separately in a clear manner, being expelled to the exterior of cavity 2 without undergoing any hydrodynamic disturbance, i.e. without cell band remixing with those immediately adjacent, thus allowing a clear and rapid individuation of cells of differing densities initially present in the cell population examined.

In order to illustrate the use of the device, the separation of cellular populations from human bone marrow will be taken as an example.

It is known that human bone marrow contains various cellular populations with differing densities (see "Haematologica", January-February 1997): some cellular types are light (low density), others are of intermediate density and others are heavy (high density). In order to separate said cellular populations and collect

them separately, a cellular separator is used as described above with reference to Figures 2 to 4, in which cavity 2 has a volume of 90ml, and the various ducts are 2mm in diameter.

90ml of liquid (with a continuous density gradient between 1.050 and 1.100g/l) is introduced into the separator device through duct 4. Then 5ml of bone marrow monodispersed cellular suspension is introduced above the liquid already present through channel 8.

The cellular separator device, thus loaded, is then centrifuged at 400 g for 1 hour, to obtain successive stratification of the different cellular populations in discrete bands of the suspension present inside cavity 2 of the device. For the separate collection of the various cellular fractions, so separated, a heavy water immiscible liquid is introduced at the base through duct 4 at a flow velocity of 2ml/min, while simultaneously pumping air from the top, through tube 8, at the same rate (2ml/min). Thus the cellular fraction of intermediate density, present at the height of the duct 9 exit holes located halfway in cavity 2, is compressed and forced out through these and is collected in a few minutes (approx. 5 minutes), without remixing with the other cellular fractions due to hydrodynamic disturbances.

Similarly, to collect the light cellular fractions situated in the upper part of the suspension, or the heavy ones placed in the lower part of the same suspension, it is sufficient to move the strata of differing densities towards the top or the bottom (using the peristaltic pump connected to ducts 4 and tube 8) so as to obtain the cellular fraction desired, at the height of the lateral exit holes (ducts 9) after which, in cavity 2, heavy liquid is introduced from the base (using the peristaltic pump) and air from the top to collect the desired cellular fraction through the lateral exit holes mentioned.

CLAIMS

1. A device for the separation of human or animal cells of different densities, from cellular dispersions in which they are contained, having a base and a top and containing an elongated chamber whose cross section decreases from the base towards the top of the device which contains at least a first channel, one end of which opens on the inside of the said chamber near the said base and the other end connectable to a pressurised liquid source and a second channel whose end opens in the same chamber at level corresponding to the device top, characterised by the fact that there is at least an additional channel in the said device, one end of which opens at an intermediate level of the said chamber length, and the other end opens outwards from the device and in that near said base a flow deflector is provided to disperse evenly through the entire cross section of the inside chamber of the incoming fluid arriving from said first channel.

2. A separation method of human or animal cells with different densities from cellular dispersions which contain them, in which, first of all, a dispersion of a cell population is introduced into the chamber of a separator device previously filled with a liquid whose density varies from a maximum, near the device base, to a minimum near the top, and the device with cellular dispersion is subjected to centrifugation for the distribution of the cells into distinct fractions with differing densities, the method being characterised by the fact that the recovery of the separated fractions takes place by introducing a very dense water immiscible liquid at the base of the separator device while simultaneously pumping a gas, at the same pressure as said very dense liquid, to the device top so that the layered cellular fractions inside the chamber are compressed and forced out through at least one hole situated at an intermediate level of said chamber length.

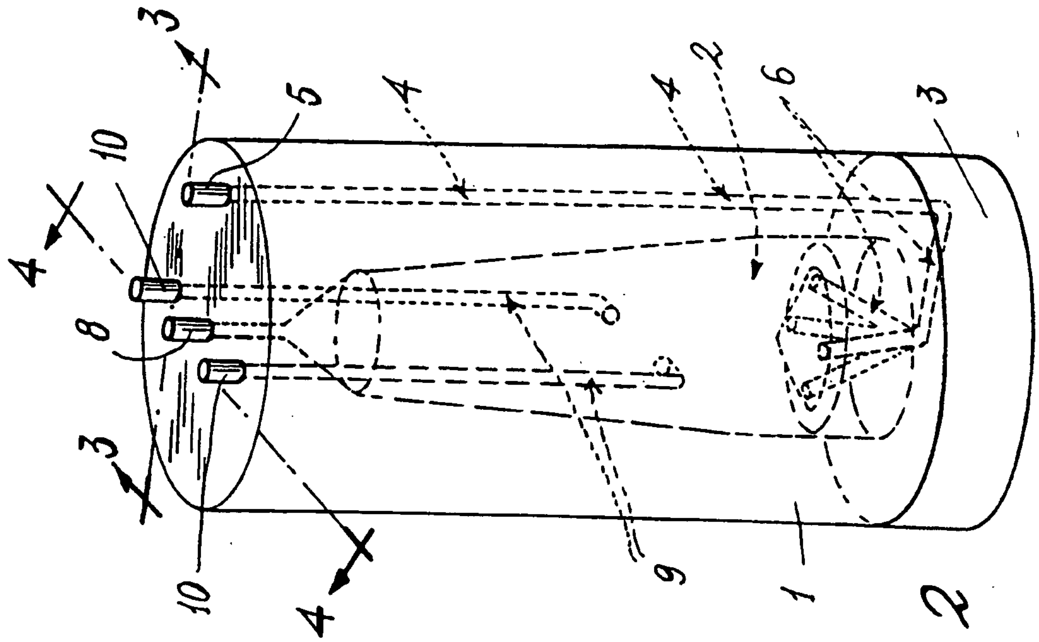
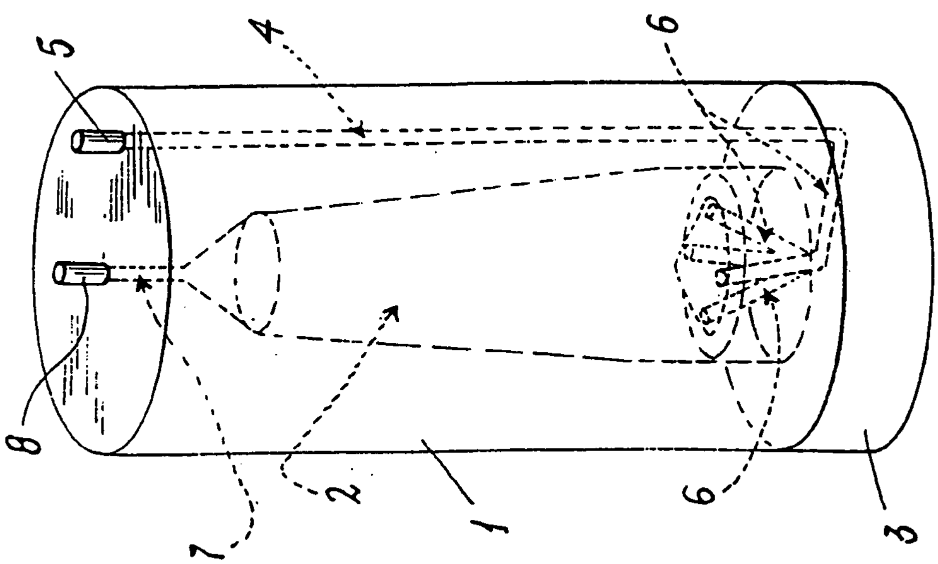


Fig. 2

Fig. 1



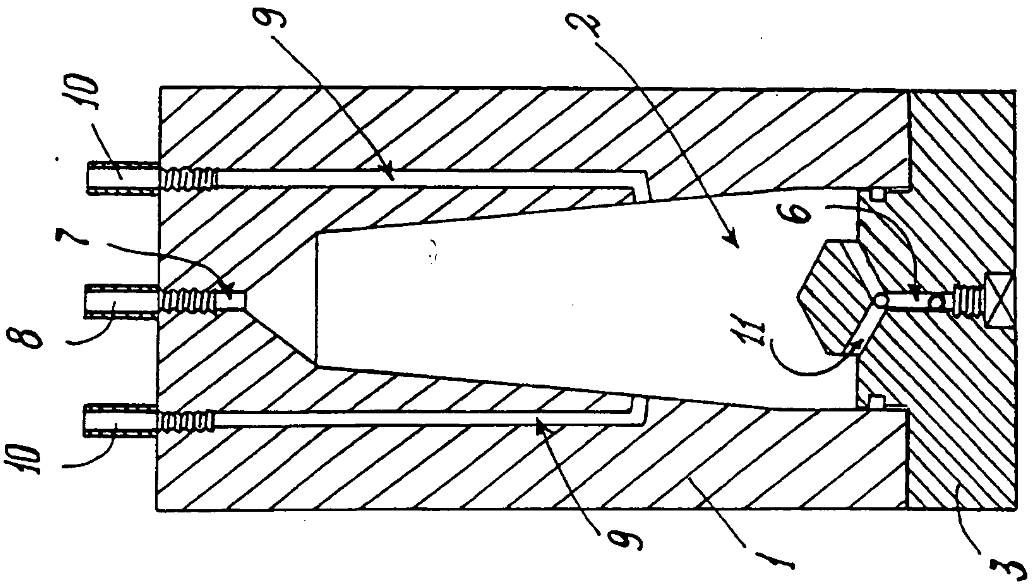


Fig. 4

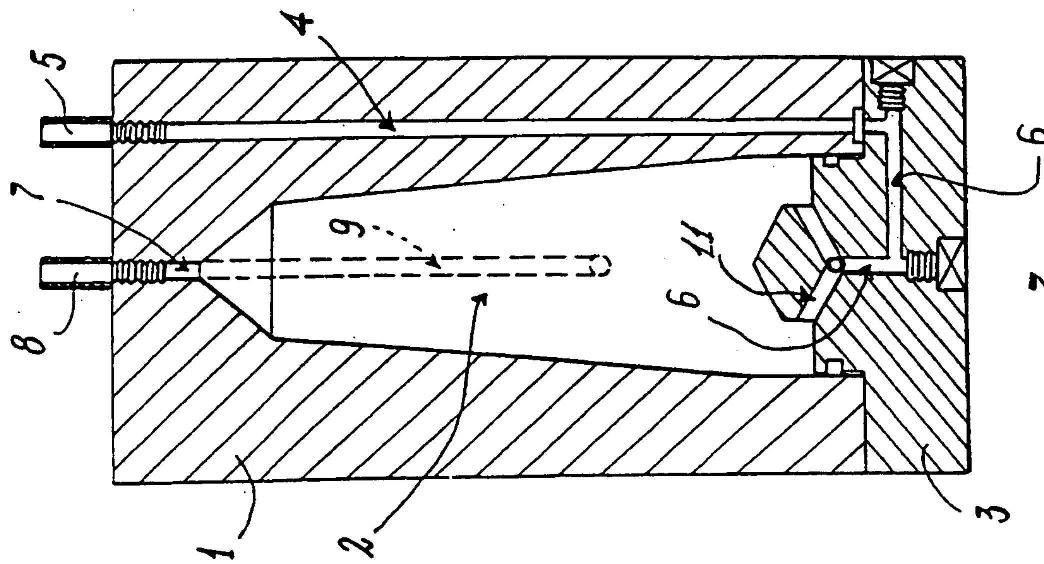


Fig. 3

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 98/06865

A. CLASSIFICATION OF SUBJECT MATTER

G 01 N 15/00,B 04 B 5/04,B 04 B 7/02,G 01 N 33/48

According to International Patent Classification (IPC) or to both national classification and IPC 6

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G 01 N,B 04 B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5100372 A (HEADLEY, T.D.) 31 May 1992 (31.05.92), abstract, column 2, lines 47-62, fig. 1. --	1,2
A	US 4146172 A (CULLIS, H.M.) 27 March 1979 (27.03.79), abstract, column 2, lines 40-55, claims, fig. 1-11. --	1,2
A	EP 0486480 A2 (BAXTER INT. INC.) 20 May 1992 (20.05.92), abstract, fig. 1,2. --	1,2
A	EP 0475457 A1 (TEIJIN LIMITED) 18 March	1,2

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 February 1999	12.03.99

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	ERBER e.h.

International Application No
PCT/EP 98/06865

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	1992 (18.03.92), abstract, page 3, line 25 - page 5, line 57, claims, fig. 1,2. --	
A	EP 0420153 A1 (TEIJIN LIMITED) 03 April 1991 (03.04.91), abstract, claims, fig. 2,3,4. --	1,2
A	EP 0198462 A2 (ASSAF PHARM. IND. LTD.) 22 October 1986 (22.10.86), abstract, page 3, line 2 - page 6, line 22, fig. 1-6. --	1,2
A	WO 96/07097 A1 (ACTIVATED CELL THERAPY, INC.) 07 March 1996 (07.03.96), abstract, claim 1, fig. 1,6. --	1,2
A	SITAR, G. et al. Physical procedures for the separation of blood and marrow cells. Haematologica, Jan.-Feb. 1989, Vol. 74, pages 95-111. --	1,2
A	TULP, A. A separation chamber to sort cells, nuclei, and chromosomes at moderate g forces. II. Studies on velocity sedimentation and equilibrium density centrifugation of mammalian cells. An International Journal of Analytical and Preparative Methods. 1981, Vol. 117, pages 354-365. ----	1,2

ANHANG / ANNEX / ANNEXE

zum internationalen Recherchenbericht über die internationale Patentanmeldung Nr.

to the International Search Report to the International Patent Application No.

au rapport de recherche international relatif à la demande de brevet international n°

PCT/EP 98/06865 SAE 215870

In diesem Anhang sind die Mitglieder der Patentfamilien der im obengenannten internationalen Recherchenbericht angeführten Patentdokumente angegeben. Diese Angaben dienen nur zur Unterrichtung und erfolgen ohne Gewähr.

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The Office is in no way liable for these particulars which are given merely for the purpose of information.

La presénte annexe indique les membres de la famille de brevets relatifs aux documents de brevets cités dans le rapport de recherche international visée ci-dessus. Les reseignements fournis sont donnés à titre indicatif et n'engagent pas la responsibilité de l'Office.

Im Recherchenbericht angeführtes Patentdokument / Patent document cited in search report / Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung / Publication date / Date de publication	Mitglied(er) der Patentfamilie / Patent family member(s) / Membre(s) de la famille de brevets	Datum der Veröffentlichung / Publication date / Date de publication
US A 5100372	31-03-92	keine - none - rien	
US A 4146172	27-03-79	CA A1 1090749	02-12-80
		DE A1 2845364	19-04-79
		DE C2 2845364	07-09-89
		DE C3 2845364	28-04-94
		FR A1 2406478	18-05-79
		FR B1 2406478	05-10-84
		GB A1 2006058	02-05-79
		GB B2 2006058	24-02-82
		JP B4 63014628	31-03-88
		JP A2 54064893	25-05-79
EP A2 486480	20-05-92	CA A1 1317917	18-05-93
		DE CO 3884871	18-11-93
		DE T2 3884871	19-05-94
		DE CO 3855891	28-05-97
		DE T2 3855891	20-11-97
		EP A1 299054	18-01-89
		EP A3 486480	22-07-92
		EP B1 299054	13-10-93
		EP A1 765687	02-04-97
		EP B1 486480	23-04-97
		JP T2 1502029	13-07-89
		JP B2 2556741	20-11-96
		US A 4834890	30-05-89
		WO A1 8805691	11-08-88
		US A 5104526	14-04-92
		US A 5316666	31-05-94
		US A 5322620	21-06-94
		US A 5370802	06-12-94
		US A 5494578	27-02-96
		US A 5529691	25-06-96
		US A 5573678	12-11-96
		US A 5628915	13-05-97
		US A 5632893	27-05-97
		US A 5641414	24-06-97
		US A 5656163	12-08-97
		US A 5693232	02-12-97
		US A 5750039	12-05-98
		US A 5792372	11-08-98
		US A 5807492	15-09-98
		US A 5849203	15-12-98
		US A 5076911	31-12-91
		US A 5316667	31-05-94
		DE CO 69319418	06-08-98
		EP A1 623044	09-11-94
		EP A4 623044	08-03-95
		EP B1 623044	01-07-98
		JP T2 7502676	23-03-95
		WO A1 9408689	28-04-94
		AU A1 3421093	28-07-93
		AU B2 663160	28-09-95
		CA AA 2103911	24-06-93
		DE CO 69223042	11-12-97
		DE T2 69223042	10-06-98
		EP A1 572656	08-12-93
		EP A4 572656	14-12-94
		EP B1 572656	05-11-97
		JP T2 6505674	30-06-94
		WO A1 9312888	08-07-93
		US A 5681273	28-10-97
		US A 5360542	01-11-94
		US A 5549834	27-08-96
		US A 5639382	17-06-97
		US A 5676841	14-10-97
		US A 5690835	25-11-97
		US A 5730883	24-03-98
		US A 5804079	08-09-98
		US A 5833866	10-11-98
		DE CO 69310342	05-06-97

		NO A 970533	04-04-97
		WO A1 9640400	19-12-96
		AU A1 58776/96	30-12-96
		AU B2 700756	14-01-99
		CA AA 2195067	19-12-96
		EP A1 775005	28-05-97
		EP A4 775005	13-01-99
		JP T2 10504043	14-04-98
		NO A0 970538	06-02-97
		NO A 970538	04-04-97
		WO A1 9640399	19-12-96
		AU A1 58778/96	30-12-96
		AU B2 692396	04-06-98
		CA AA 2195069	19-12-96
		EP A1 775010	28-05-97
		JP T2 10507204	14-07-98
		NO A0 970537	06-02-97
		NO A 970537	04-04-97
		WO A1 9640401	19-12-96
EP A1 475457	18-03-92	JP A2 3175980	31-07-91
		JP B4 7008236	01-02-95
		DE C0 69029887	20-03-97
		DE T2 69029887	22-05-97
		EP A1 420153	03-04-91
		EP B1 420153	05-02-97
		US A 5250432	05-10-93
		DE C0 3684898	21-05-92
		DE C0 3650550	05-09-96
		DE T2 3650550	09-01-97
		EP A2 229289	22-07-87
		EP A3 229289	08-02-89
		EP A1 480245	15-04-92
		EP B1 229289	15-04-92
		EP B1 480245	31-07-96
		JP A2 62134086	17-06-87
		JP B4 4025796	01-05-92
EP A1 420153	03-04-91	JP A2 3175980	31-07-91
		JP B4 7008236	01-02-95
		DE C0 69029887	20-03-97
		DE T2 69029887	22-05-97
		EP A1 475457	18-03-92
		EP B1 420153	05-02-97
		US A 5250432	05-10-93
		DE C0 3684898	21-05-92
		DE C0 3650550	05-09-96
		DE T2 3650550	09-01-97
		EP A2 229289	22-07-87
		EP A3 229289	08-02-89
		EP A1 480245	15-04-92
		EP B1 229289	15-04-92
		EP B1 480245	31-07-96
		JP A2 62134086	17-06-87
		JP B4 4025796	01-05-92
EP A2 198462	22-10-86	AT E 84992	15-02-93
		CA A1 1306985	01-09-92
		DE C0 3687588	11-03-93
		DE T2 3687588	19-05-93
		EP A3 198462	02-11-88
		EP B1 198462	27-01-93
		IL A0 74967	30-08-85
		IL A1 74967	31-10-88
		JP A2 62000836	06-01-87
		US A 4824560	25-04-89
WO A1 9607097	07-03-96	AU A1 35025/95	22-03-96
		AU B2 700743	14-01-99
		CA AA 2198607	07-03-96
		EP A1 778944	18-06-97
		JP T2 10508190	18-08-98
		NZ A 292756	28-10-98
		US A 5474687	12-12-95
		US A 5663051	02-09-97
		US A 5789148	04-08-98
		US A 5646004	08-07-97
		US A 5648223	15-07-97
		US A 5840502	24-11-98
		AU A1 14150/97	03-07-97
		CA AA 2239725	19-06-97
		WO A1 9721488	19-06-97

DE	T2	69310342	13-11-97
EP	A1	618831	12-10-94
EP	A4	618831	29-03-95
EP	A2	749771	27-12-96
EP	A2	755708	29-01-97
EP	B1	618831	02-05-97
EP	A3	749771	01-04-98
EP	A3	755708	01-04-98
JP	T2	7502446	16-03-95
WO	A1	9408687	28-04-94
AU	A1	59368/96	30-12-96
AU	B2	701731	04-02-99
CA	AA	2221731	19-12-96
EP	A1	851784	08-07-98
EP	A4	851784	23-12-98
NO	A0	975684	05-12-97
NO	A	975684	02-02-98
WO	A1	9640406	19-12-96
AU	A1	62497/96	30-12-96
AU	B2	694053	09-07-98
CA	AA	2195187	19-12-96
EP	A1	775011	28-05-97
JP	T2	10503712	07-04-98
NO	A0	970535	06-02-97
NO	A	970535	02-04-97
WO	A1	9640404	19-12-96
AU	A1	59356/96	30-12-96
AU	B2	694047	09-07-98
CA	AA	2195188	19-12-96
EP	A1	775007	28-05-97
EP	A4	775007	25-03-98
JP	T2	10504243	28-04-98
NO	A0	970536	06-02-97
NO	A	970536	02-04-97
WO	A1	9640402	19-12-96
AU	A1	59357/96	30-12-96
AU	B2	694366	16-07-98
CA	AA	2195070	19-12-96
EP	A1	775008	28-05-97
EP	A4	775008	29-04-98
JP	T2	10506829	07-07-98
NO	A0	970532	06-02-97
NO	A	970532	02-04-97
WO	A1	9640403	19-12-96
DE	C0	69310711	19-06-97
DE	T2	69310711	27-11-97
EP	A1	619752	19-10-94
EP	A4	619752	29-03-95
EP	B1	619752	14-05-97
JP	T2	7502447	16-03-95
WO	A1	9408688	28-04-94
DE	C0	69305494	21-11-96
DE	T2	69305494	28-05-97
EP	A1	618830	12-10-94
EP	A4	618830	29-03-95
EP	B1	618830	16-10-96
JP	T2	7502449	16-03-95
WO	A1	9408692	28-04-94
EP	A1	618829	12-10-94
EP	A4	618829	20-09-95
JP	T2	7502677	23-03-95
WO	A1	9408690	28-04-94
CA	AA	2143832	02-02-95
EP	A1	666771	16-08-95
EP	A4	666771	16-09-98
JP	T2	8502440	19-03-96
WO	A1	9503112	02-02-95
AU	A1	59369/96	30-12-96
AU	B2	701805	04-02-99
CA	AA	2195191	19-12-96
EP	A1	773811	21-05-97
JP	T2	10503697	07-04-98
NO	A0	970543	06-02-97
NO	A	970543	04-04-97
WO	A1	9640353	19-12-96
CA	AA	2143830	02-02-95
EP	A1	662857	19-07-95
EP	A4	662857	23-04-97
EP	A2	882464	09-12-98
EP	A2	882465	09-12-98
JP	T2	8502439	19-03-96
WO	A1	9503107	02-02-95
AU	A1	59359/96	30-12-96
AU	B2	692711	11-06-98
CA	AA	2195071	19-12-96
EP	A1	775009	28-05-97
JP	T2	10503696	07-04-98
NO	A0	970542	06-02-97
NO	A	970542	04-04-97
WO	A1	9640405	19-12-96
AU	A1	58777/96	30-12-96
AU	B2	694045	09-07-98
CA	AA	2195068	19-12-96
EP	A1	775006	28-05-97
JP	T2	10503695	07-04-98
NO	A0	970533	06-02-97